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Lindane uptake and degradation by aquatic *Streptomyces* sp. strain M7

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Abstract

Five actinomycete strains isolated from pesticide-contaminated sediments were able to grow in the presence of 10 $\mu g\,l^{-1}$ lindane, an organochlorine pesticide. The strain growing best in the presence of lindane as the only carbon source was identified as *Streptomyces* sp. M7. After 96 h of incubation in synthetic medium containing lindane and glucose, both substrates were simultaneously consumed; glucose 6.0 $g\,l^{-1}$ improved lindane degradation and obtained biomass. When *Streptomyces* sp. M7 was cultured in presence of lindane plus glucose, the disappearance of the pesticide from the medium and the lindane degradation was observed after 72 h of incubation. This is the first report of lindane degradation without intracellular accumulation or biotransformation products of lindane using *Streptomyces* sp. under aerobic conditions.

Relevance to industry: This is the first report of lindane removal without intracellular accumulation or biotransformation products of lindane using *Streptomyces* sp. strain M7, an actinomycete isolated from pesticide-contaminated sediments from Tucuman, Argentina.

Keywords: *Streptomyces*; Lindane; γ-hexachlorocyclohexane; Degradation; Bioremediation

1. Introduction

Lindane, or γ-hexachlorocyclohexane (γ-HCH), has been extensively used worldwide for the control of agricultural and medical pests. Due to widespread use and persistence, it is a common pollutant found ubiquitous in the biosphere (Zuloaga et al., 2000; Okeke et al., 2002). This insecticide is known for its tendency to bioaccumulate and its toxicity to non-target organisms, including humans. (De Cruz et al., 1996; Johri et al,. 1996). The low aqueous solubility and chlorinated nature of lindane contribute to its persistence and resistance to degradation by microorganisms (Phillips et al., 2001). There have been reports on the occurrence of γ-HCH residues in soil, water, air, plants, agricultural products, animals, food, microbial environment, and humans (Albanis et al., 1998; Hura et al., 1999; Chaile et al., 1999; Waite et al., 2001; Botella et al., 2004). γ-HCH is a lipophilic compound, and therefore tends to accumulate and concentrate in the body fat of man (Johri et al., 2000). Since the toxicity of γ-HCH is well established, it is imperative to develop methods by which lindane can be removed from the environment and to search for new bioremediation agents for these compounds (Singh and Kuhad, 1999).

There have been some reports regarding aerobic degradation of γ-HCH by Gram-negative bacteria like *Sphingomonas* (Singh et al., 1999) and by the white-rot fungi *Trametes hirsutus, Phanerochaete chrysosporium, Cyathus bulleri* and *Phanerochaete sordida* (Mougin et al., 1999; Singh and Kuhad, 1999, 2000). However, little information is available on the ability of organochlorine pesticide biotransformation by Gram-positive microorganisms and particularly by actinomycete species, the main group of bacteria present in soils and sediments (De Schrijver and De Mot, 1999). These Gram-positive microorganisms have a great potential for biodegradation of organic and inorganic toxic compounds, and also could remove different organochlorine pesticides when other

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carbon sources are present in the medium as energy source (Amoroso et al., 1998; Ravel et al., 1998; Benimeli et al., 2003). However, the ability of actinomycetes to transform organochlorine pesticides has not been widely investigated, despite studies demonstrating that actinomycetes, specifically of the genus *Streptomyces*, have been able to oxidize, partially dechlorinate and dealkylate aldrin, DDT and herbicides like metolachlor or atrazine (Ferguson and Korte, 1977; Liu et al., 1990, 1991; Radosevich et al., 1995).

The use of indigenous actinomycete strains for bioremediation of soils is an attractive approach, since these microorganisms are already adapted to the habitat. In addition to their potential metabolic diversity, strains of *Streptomyces* may be well suited for soil inoculation as a consequence of their mycelial growth habit, relatively rapid rates of growth, colonization of semi-selective substrates and their ability to be genetically manipulated (Shelton et al., 1996). One additional advantage is that the vegetative hyphal mass of these microorganisms can differentiate into spores that assist in spread and persistence; the spores are a semi-dormant stage in the cycle life that can survive in soil for long periods (Mayfield et al., 1972; Ensign, 1978) and impart resistance to low nutrient concentrations and water availability (Karagouni et al., 1993).

Presence of lindane ($2.0\,\mu g\,l^{-1}$), methoxychlor ($1.3\,\mu g\,l^{-1}$), aldrin and dieldrin (which did not exceed $0.03\,\mu g\,l^{-1}$) were detected in the main hydrographic system of the Tucumán state, the Salí river (Chaile et al., 1999). In order to establish a strategy for bioremediation, samples of river sediments and from other local sources were collected to isolate and select wild-type streptomycete strains with the ability to tolerate and remove organochlorine pesticides (Benimeli et al., 2003). Continuing with the previous study, the aim of the present work is to select actinomycete strains with high capability of lindane degradation in liquid cultures and to study the effect of glucose on pesticide detoxification.

2. Materials and methods

2.1. Microorganisms and culture conditions

Twenty-two actinomycete strains isolated from wastewater sediment samples of a copper filter plant (contaminated site), and six from El Cadillal Lake (non-contaminated site) previously tested on the basis of its multiple tolerance to 11 organochlorine pesticides (Benimeli et al., 2003) were selected for the present work.

2.2. Screening of actinomycete strains for the capacity to grow in the presence of lindane

Spore suspension of the 28 actinomycete strains were inoculated in a liquid minimal medium (MM), containing (grams per liter): L-asparagine, 0.5; K_2HPO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.20; $FeSO_4 \cdot 7H_2O$, 0.01 (Hopwood, 1967). Lindane (ULTRAScientific, North Kingstown, RI) was dissolved in methanol (HPLC grade, Merck, Argentina), sterilized by passing through a 0.22 μm pore size Millipore filter and then added aseptically to the autoclaved medium to a final concentration of $10\,\mu g\,l^{-1}$. All cultures were incubated on a rotatory shaker (100 rpm) at 30 °C, for 72 h. Supernatant samples of centrifuged cultures ($9900 \times g$, 30 min, 4 °C) were used to determine residual lindane by gas chromatography. Biomass was estimated after centrifugation by washing the pellets with 25 mM Tris-EDTA buffer (pH 8.0) and drying to constant weight at 105 °C.

2.3. Lindane analysis by gas chromatography

Lindane in cell-free supernatant samples ($9900 \times g$, 30 min, at 4 °C) were extracted by solid phase extraction (SPE) using C18 columns, and was quantified in a gas chromatograph (Hewlett-Packard 6890, Wilmington, DE) equipped with a HP 5 capillary column (30 m × 0.53 mm × 0.35 m) and ^{63}Ni ECD detector, split/splitless injector HP 7694 and ChemStation Vectra XM software. Quantitative analysis of samples was performed using appropriate lindane calibration standards (ULTRA-Scientific, North Kingstown, RI).

2.4. Growth determination in presence of lindane in five selected actinomycete strains

Spore suspensions of five selected actinomycete strains, M2, M4, M5, M7 and M14, were cultured in flasks containing 30 ml MM supplemented with lindane $10\,\mu g\,l^{-1}$, on a rotatory shaker (100 rpm) at 30 °C, for 96 h. Samples were taken each 24 h and centrifuged ($9900 \times g$, 10 min). Biomass was determined as mentioned before. Control cultures in MM without added lindane were done.

2.5. 16S ribosomal RNA (rRNA) sequencing

Total DNA was prepared from strain M7 according to the method described by Hoffman and Winston (1987). Oligonucleotide primers with specificity for eubacterial 16S rRNA genes (forward primer 8–27: 5′-AGA GTT TGA TCC TGG CTC AG-3′, Weisburg et al., 1991, and reverse primer 1389: 5′-ACG GGC GGT GTG TAC AAG- 3′, Marchesi et al., 1998) were used to amplify 16S rDNA. PCR fragments were purified using Qiaquick Gel Extraction Kit (Qiagen, Valencia, CA) and sequenced using the PRIMS READY reaction Kit (PE Applied BioSystems, Foster City, CA) and an ABI 373A sequencer (PE Applied BioSystems, Foster City, CA).

Sequencing data were analyzed by comparison to 16S rRNA genes in the Ribosomal Database Project and EMBL-GeneBank databases, and aligned manually with the Phydit software (Chun, 1985). The 16S rRNA gene sequence of strain M7 had been deposited in GeneBank under accession number AY459531.

An evolutionary tree was constructed using the neighbor-joining Fitch-Margoliash (1967) algorithm in the PHYLIP package (Felsenstein, 1993). Evolutionary distances matrices for the Fitch-Margoliash method were generated as described by Jukes and Cantor (1969). The resultant tree topologies were evaluated by performing bootstrap analyses of the neighbor-joining data (Felsenstein, 1985) based on 40,000 resamplings.

2.6. Precultivation in lindane or glucose effect on the capacity of Streptomyces M7 to remove lindane

Streptomyces sp. M7 was precultured in MM containing lindane ($10\,\mu g\,l^{-1}$) or glucose ($6\,g\,l^{-1}$) for 72 h. Cultivation was carried out in Erlenmeyer flasks containing 150 ml medium, at 30 °C on a rotatory shaker (100 rpm). Cells were centrifuged at $9900 \times g$ for 10 min, washed with 25 mM Tris-EDTA buffer (pH 8.0), and then inoculated in MM supplemented with lindane ($10\,\mu g\,l^{-1}$), or lindane ($10\,\mu g\,l^{-1}$) plus glucose ($6\,g\,l^{-1}$), and cultivated for 96 h. Biomass and residual lindane were analyzed in samples every 24 h. To evaluate the effect of glucose concentration similar experiments were carried out in MM supplemented with $0.6\,g\,l^{-1}$ of glucose.

2.7. Intracellular lindane determination

Lindane uptake and its intracellular accumulation by *Streptomyces* M7 were analyzed after preculturing in lindane or glucose. The precultured cells were centrifuged at 9900 × *g* for 10 min and washed in 25 mM Tris-EDTA buffer (pH 8.0), before inoculation in MM supplemented with lindane ($10\,\mu g\,l^{-1}$) and glucose ($6\,g\,l^{-1}$). The microorganisms were cultivated on a rotatory shaker (100 rpm) at 30 °C, for 96 h. Samples were taken every 24 h, filtered and washed with ethyl acetate and distilled water to remove lindane that is strongly bound. The presence of lindane in the mycelium was checked by extraction of mycelial material with ethyl acetate and determined by gas chromatography (Singh and Kuhad, 1999; Benimeli et al., 2004).

2.8. Statistical analysis

Each experiment was done in triplicate and the results are arithmetic means. For statistical evaluation, Student's *t*-test and One-way ANOVA for the analysis of variance were used.

3. Results and discussion

3.1. Screening of actinomycete strains for the capacity to grow in the presence of lindane

In a study of the water quality of Salí River (Tucumán, Argentina), which flows parallel of the wastewater of a copper filter plant (contaminated site), presence of $0.2–2.0\,\mu g\,l^{-1}$ of lindane was detected (Chaile et al., 1999). On the other hand, in sediment samples of this contaminated site, $90–97\,\mu g\,l^{-1}$ of lindane was measured (Benimeli, 2004). According to these results, a $10\,\mu g\,l^{-1}$ lindane concentration was selected in order to select actinomycete strains with the ability to grow in the presence of the pesticide.

Twenty-eight pesticide tolerant wild-type actinomycete strains were selected to determine the capacity of growth in the presence of lindane $10\,\mu g\,l^{-1}$ in liquid medium (Benimeli et al., 2003).

Two criteria of analysis were established. The first criterion was the determination of residual lindane concentration, in order to establish the population behavior of the studied microorganisms. Strains showing values of residual lindane higher than the average were eliminated from the study. The second criterion was to establish the ration of residual lindane concentration and microbial growth; the strains were selected for which these values were minimum.

All actinomycete strains isolated from non-contaminated sites showed comparatively high residual lindane concentrations and were eliminated from the study. Of the 22 microorganisms isolated from contaminated sediment samples, seven were found to leave residual lindane concentrations higher than the average value. Finally five strains, M2, M4, M5, M7 and M14, isolated from the polluted site, were selected as the most efficient to growth in the presence of lindane as the only carbon source (Table 1). These microorganisms left residual lindane concentrations lower than the population average and had minimal ratios between residual lindane concentration and microbial growth.

Table 1
Selection of five actinomycete strains with capacity to grow in the presence of lindane $10\,\mu g\,l^{-1}$

Strains	Dry weight ($mg\,ml^{-1}$)	Residual lindane ($\mu g\,l^{-1}$)	Residual lindane/dry weight
M2	0.59	1.33	2.25×10^{-6}
M4	0.45	2.60	5.77×10^{-6}
M5	0.38	2.00	5.26×10^{-6}
M7	0.35	1.20	3.43×10^{-6}
M14	0.48	3.29	6.85×10^{-6}

Residual lindane average: $4.60\,\mu g\,l^{-1}$.

The actinomycetes isolated from the contaminated samples showed efficient growth with lindane as the only carbon source, indicating that these microorganisms could survive in the contaminated environments either due to the tolerance to the pesticides or due to their ability to degrade them. It is important to notice that these actinomycete strains were not able to grow in MM containing L-asparagine as a sole carbon source (Benimeli et al., 2003). On the other hand, there was no evidence of microbial growth in control cultures in MM without added lindane.

For the five selected strains, a time course of growth in presence of lindane as the only carbon source, was performed (Fig. 1). Strain M7 showed the best growth during the 96 h of incubation in presence of lindane. At 24 h of incubation, M7 reached twice the growth of strains M5 and M14. Based on the growth curves of Fig. 1, strain M7 was selected for further study.

3.2. Phylogenetic analysis of strain M7

Macroscopic and microscopic observations and chemotaxonomic analysis of strain M7 indicated its belonging to the genus *Streptomyces* (Benimeli et al., 2003). Using specific primers for PCR, 1313 bp of 16S rDNA were obtained and sequenced. Analysis of the 16S rDNA sequence confirmed the placement of this strain with the genus *Streptomyces* (99% homology). *Streptomyces* sp. M7 was closely related to unidentified species of *Streptomyces* sp. CHR28. To our knowledge, *Streptomyces tendae* is the closest relative identified species to *Streptomyces* sp. M7.

3.3. The effect of precultivation in lindane or glucose on the capacity of Streptomyces M7 to remove lindane

Because *Streptomyces* M7 grew poorly when it was two successive times cultured in lindane as the only carbon source, it was evaluated if the addition of glucose could improve microbial growth and lindane removal.

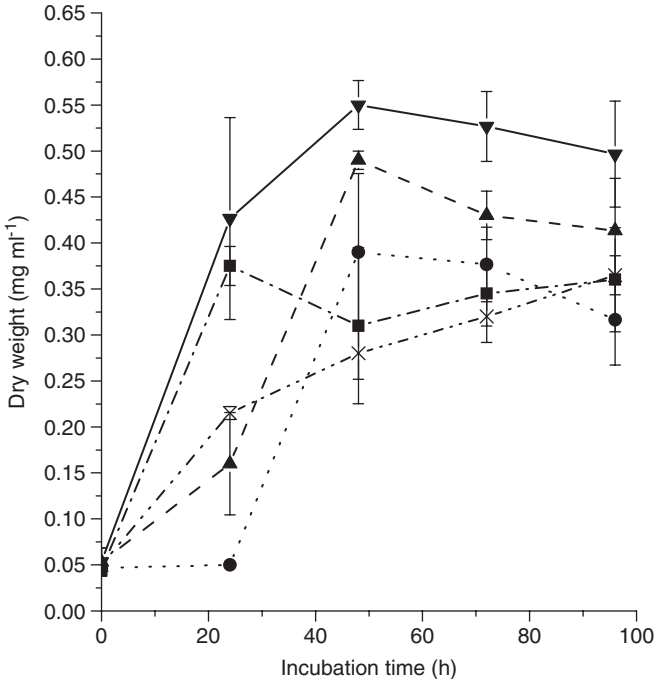

Fig. 1. Growth of five actinomycete strains in the presence of lindane ($10\,\mu g\,l^{-1}$). Strains: M2 (●); M4 (■); M5 (▲); M7 (▼) and M14 (×). Error bars represent standard deviations.

The disappearance of γ-HCH was studied after preculturing the microorganisms in media containing lindane or glucose, followed by cultivation in medium supplemented only with γ-HCH ($10\,\mu g\,l^{-1}$). Growth kinetics of *Streptomyces* M7 in medium supplemented with lindane as the only carbon source revealed a stationary state between 24 and 48 h, followed by an increase of biomass and concomitant decrease of residual lindane in the culture (Fig. 2a). On the other hand, *Streptomyces* M7 growth cultured first in medium containing glucose reached about the same biomass in the first 24 h, but followed by 27% of biomass reduction (Fig. 2b). The abrupt decrease in dry weight after 24 h of growth is not correlated with an increase of lindane in the supernatant, indicating that lindane had previously not simply been sequestered in the cells to be released again after cell lysis, but rather degradaded during the first 24 h of growth. Residual lindane concentration in the medium decreased only about 20% in the first 24 h of culture, but after 48 h kept almost constant in *Streptomyces* M7 precultured in glucose followed by lindane cultivation (Fig. 2b).

In the next experiment, the microorganism was precultured in medium containing lindane $10\,\mu g\,l^{-1}$ or alternatively glucose $6.0\,g\,l^{-1}$, followed by cultivation in medium supplemented with both lindane and low glucose concentration (Fig. 3). When *Streptomyces* M7 was precultured in lindane (Fig. 3a) simultaneous removal of both substrates was observed in the first 48 h, when 50% of lindane and 100% of glucose had been removed from the medium. Then, lindane removal as well as the microbial growth ceased, with $0.42\,mg\,ml^{-1}$ resulting biomass obtained. When *Streptomyces* M7 grew in the presence of glucose plus lindane after preculturing in glucose (Fig. 3b), again both substrates were used during the first 48 h of incubation, with 30% lindane and 100% glucose removal. However, lindane decrease continued parallel to microbial growth, reaching 42% removal at the end of the 96 h

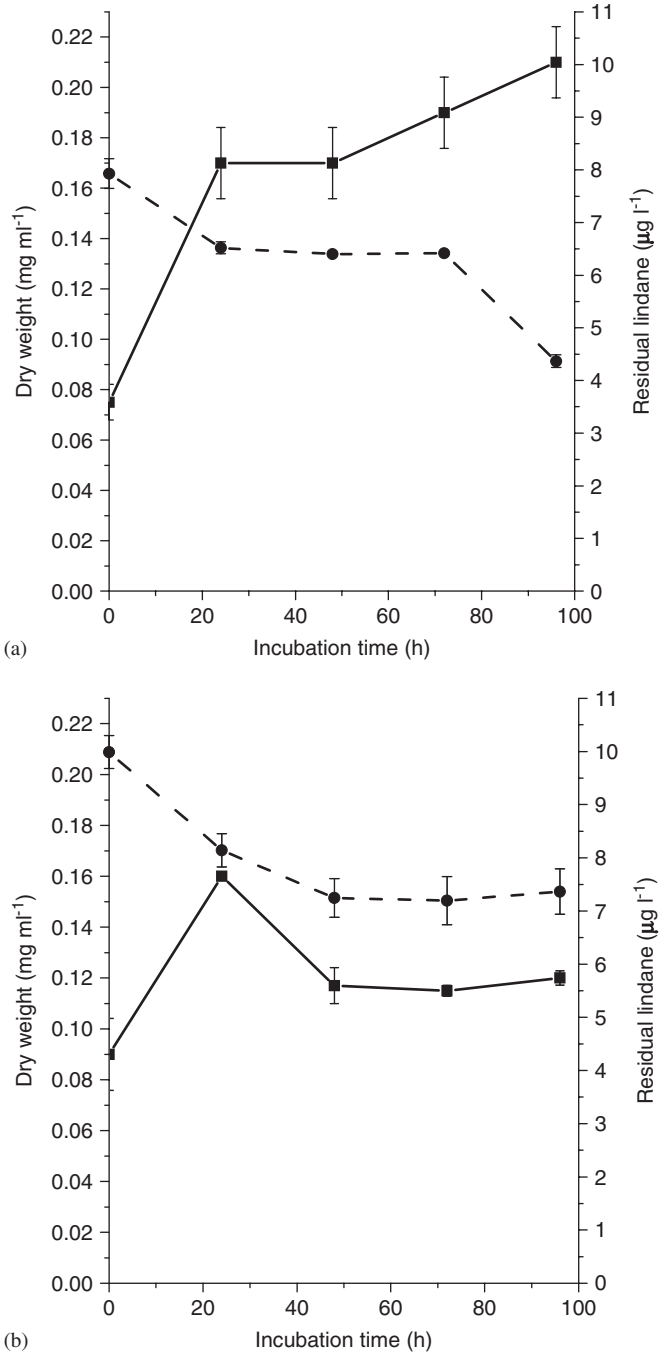

Fig. 2. Determination of growth and residual lindane in the supernatant of a $10\,\mu g\,l^{-1}$ lindane-containing culture of *Streptomyces* M7 precultured in (a) lindane and (b) glucose. Dry weight (■); residual lindane concentration (●). Error bars represent standard deviations.

incubation, when 0.36 g l^{-1} biomass had been produced (Fig. 3b).

In a further experiment, *Streptomyces* M7 was cultured in the same conditions as before but with glucose 6.0 g l^{-1} instead of 0.6 g l^{-1}. When the cells were precultured in glucose or lindane, the microbial growth and the residual lindane concentration did not show significant statistical differences ($p > 0.05$) (Fig. 4). However, the kinetic of glucose consumption showed different profiles in both cultures. After lindane precultivation, glucose consumption almost paralleled the lindane removal. Also, residual lindane remained constant after glucose exhaustion at 20% residual lindane concentration, suggesting energy-dependent process for lindane uptake by the cells (Fig. 4a). In contrast, glucose utilization after precultivation in glucose, showed a kinetic associated to the cellular growth, while lindane disappearance remained constant (Fig. 4b). After 96 h of cultivation the final concentration of residual lindane remained approximately the same in both cultures, but the rate of lindane removal was higher in the culture adapted to lindane, suggesting some degree of cell metabolism adaptability to the pesticide.

In addition, at 96 h of cultivation, residual lindane was half the amount in cultures supplemented simultaneously

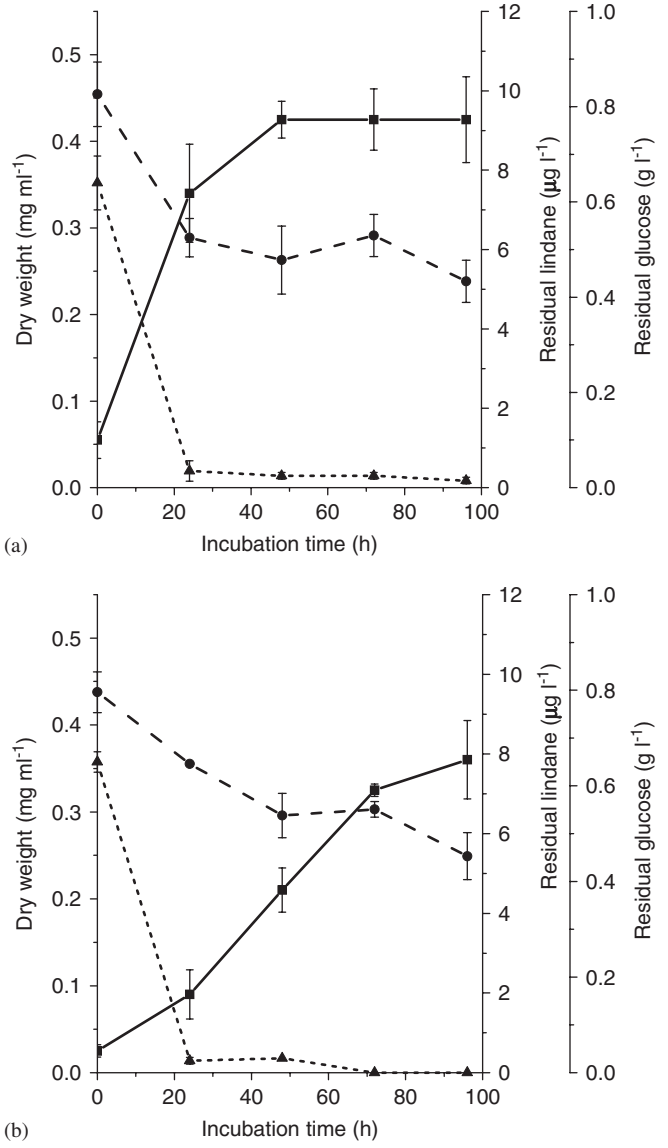

Fig. 3. Determination of growth, residual lindane and residual glucose in the supernatant of a 0.6 g l^{-1} glucose and 10 µg l^{-1} lindane-containing culture of *Streptomyces* M7 precultured in (a) lindane and (b) glucose. Dry weight (■); residual lindane concentration (●); residual glucose concentration (▲). Error bars represent standard deviations.

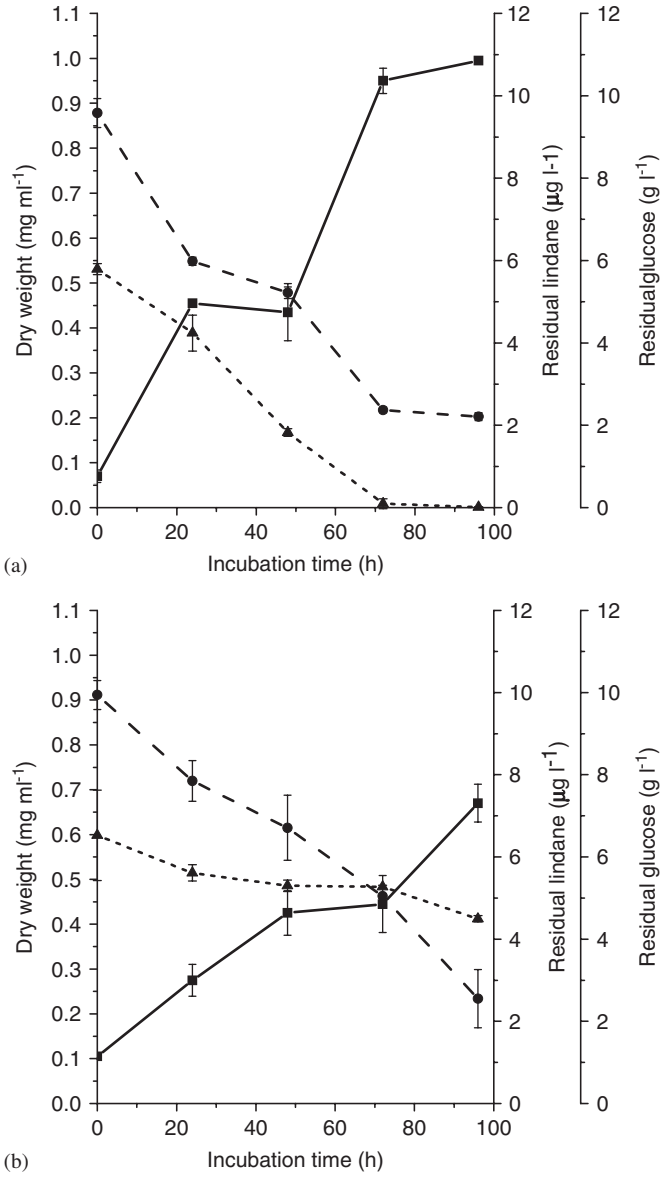

Fig. 4. Determination of growth, residual lindane and residual glucose in the supernatant of a 6 g l^{-1} glucose and 10 µg l^{-1} lindane-containing culture of *Streptomyces* M7 precultured in (a) lindane and (b) glucose. Dry weight (■); residual lindane concentration (●); residual glucose concentration (▲). Error bars represent standard deviations.

with glucose–lindane compared to cultures supplemented with lindane only (Figs. 4a, b, 2a and b). These results suggest the importance of supplementing the culture with an additional source of carbon/energy for lindane degradation.

Analysis of *Streptomyces* M7 growth first adapted in medium containing glucose or alternatively lindane, followed by cultivation in medium supplemented with lindane and different concentrations of glucose (0.6 versus 6.0 $g\,l^{-1}$) (Figs. 3 and 4) allowed the conclusion that glucose and lindane were simultaneously consumed. Presence of high glucose concentration increased the lindane removal and biomass yield.

Similar results were reported for the degradation of the herbicide metolachlor by an actinomycete specie in the presence of sucrose (Krause et al., 1985). Esposito et al. (1998) reported that actinomycete strain CCT 4916 grew poorly when glucose was omitted and diuron supplied as carbon/nitrogen source in the culture medium. Co-metabolism in the presence of a carbon source occurs in actinomycetes, and it has been shown that most pesticides can serve as phosphorous, carbon, and/or nitrogen source via partial transformation reactions. Based on many studies in different microorganisms, it can be assumed that co-metabolism is probably the most widespread mechanism for pesticide degradation (De Schrijver and De Mot, 1999).

3.4. Intracellular lindane determination in Streptomyces M7 precultured in lindane or glucose

Intracellular lindane was determined in mycelium grown on lindane plus glucose (6 $g\,l^{-1}$) after precultivation in lindane versus glucose (Table 2). Sample analysis by GC revealed only a single peak, which corresponds to lindane, from 24 h of incubation (data not shown). This result could indicate that no biotransformation occurs but maybe a complete degradation under the conditions studied. Further studies with different GC conditions are needed to rule out other metabolites in the γ-HCH pathway.

When *Streptomyces* M7 was first cultivated in lindane, almost 50% lindane was removed from the medium within 72 h, compared with 41% after glucose precultivation. However, there were no significative differences ($p > 0.05$) in lindane removal and degradation when *Streptomyces* M7 was precultivated in lindane or glucose. The GC analysis of mycelial biomass could show that the pesticide did not accumulate inside the microbial cell to significant amounts, and lindane was degraded gradually during 96 h of incubation.

On the contrary, *Phaneroachaete chrysosporium* and *Trametes hirsutus* showed ability for uptake and accumulation of lindane from the medium, but no further degradation of the intracellular pesticide was observed (Singh and Kuhad, 1999). However, in a further report, the white-rot fungi *Cyanthus bulleri* and *Phaneroachaete sordida* were reported to perform partial degradation of lindane (Singh and Kuhad, 2000). The advantage of *Streptomyces* M7 thus is that it not only accumulates lindane in the biomass, but shows high potential for degradation of the pesticide without intermediate products, which in some cases are more toxic and refractory to further degradation than the original pesticides (De Schrijver and De Mot, 1999).

Table 2
Percentage of removed and degraded lindane in the culture of *Streptomyces* M7, precultured in glucose (6 $g\,l^{-1}$) or lindane (10 $\mu g\,l^{-1}$)

Incubation time (h)	% Removed[a]		% Degraded[b]	
	Precultured in			
	Glucose	Lindane	Glucose	Lindane
24	21.6±1.6[c]	16.2±1.8	12.0±2.2	1.2±0.6
48	25.8±1.2	21.6±1.2	21.6±1.2	9.0±1.0
72	41.3±1.8	48.5±1.2	36.5±1.8	42.5±1.2
96	44.9±1.2	49.1±1.8	40.7±1.2	44.3±1.2

[a] % initial lindane − % residual lindane.
[b] % removed lindane − % intracellular lindane.
[c] Arithmetic mean ± standard deviation.

In previous studies, only two Gram-negative bacteria, *Sphingomonas paucimobilis* and *Pandoraea* sp., were described as microorganisms degrading γ-HCH under aerobic conditions (Nagata et al., 1999; Siddique et al., 2002). In addition, biodegradation of lindane by Gram-positive microorganisms was described under anaerobic conditions, performed by *Clostridium* sp. as well as some facultative anaerobic members of the *Bacillaceae* family (Jagnow et al., 1977). For the first time, the degradation of lindane by Gram-positive *Streptomyces* sp. under aerobic conditions was reported.

In conclusion, the aquatic *Streptomyces* M7 presents excellent potential for bioremediation of lindane-contaminated environments based on its ability to take up and degrade lindane from aqueous medium. Further research is necessary in order to elucidate the lindane biodegradation pathway used by *Streptomyces* M7.

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